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Effect of electromagnetic radiation on redox status, acetylcholine esterase activity and cellular damage contributing to the diminution of the brain working memory in rats

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ABSTRACT

Behavioral impairments are the most pragmatic outcome of long-term mobile uses but the underlying causes are still poorly understood. Therefore, the Aim of the present study to determine the possible mechanism of mobile induced behavioral alterations by observing redox status, cholinesterase activity, cellular, genotoxic damage and cognitive alterations in rat hippocampus. This study was carried out on 24 male Wistar rats, randomly divided into four groups (n = 6 in each group): group I consisted of sham-exposed (control) rats, group II-IV consisted of rats exposed to microwave radiation (900 MHz) at different time duration 1 h, 2 h, and 4 h respectively for 90 days. After 90 days of exposure, rats were assessing learning ability by using T-Maze. A significantly increased level of malondialdehyde (MDA) with concomitantly depleted levels of superoxide dismutase (SOD), catalase (CAT) and redox enzymes (GSH, GPX, GR, GST, G-6PDH) indicated an exposure of mobile emitted EMR induced oxidative stress by the depleted redox status of brain cells. The depletion in the acetylcholinesterase (AChE) level reveals altered neurotransmission in brain cells. Resultant cellular degeneration was also observed in the radiation-exposed hippocampus. Conclusively, the present study revealed that microwave radiation induces oxidative stress, depleted redox status, and causes DNA damage with the subsequent reduction in working memory in a time-dependent manner. This study provides insight over the associative reciprocity between redox status, cellular degeneration and reduced cholinergic activity, which presumably leads to the behavioral alterations following mobile emitted electromagnetic radiation.

1. Introduction

The effects of mobile emitting electromagnetic radiation on human health have become the subject of intense research among scientists due to the rapid increase in their use. Mobile emitted microwave radiations not only affect the brain but also cause deterioration in behavioral and cognitive processes (Cassel et al., 2004). Recent studies conducted on possible biological effects of EMR on various animal models have shown considerable negative impacts on cellular functions (Tkalec et al., 2013; Sarapultseva et al., 2014). There are also some reports showing increased risk for human health due to long-duration mobile phone use (Cardis et al., 2011; Hardell et al., 2018) Furthermore, the categorization of mobile emitted electromagnetic radiation in a possible human carcinogen 2B by the International Agency for Research on Cancer (IARC) also increases the interest of scientific society on this issue.

Recent studies reported oxidative stress is a major contributor to EMR exposure (Yurekli et al., 2006; Consales et al., 2012; Yuksel et al., 2016). Contributing factor for generation of reactive oxygen species (ROS) presumed to be altered redox metabolic status which is responsible for normal functioning of the cell under physiological conditions. The human brain is predominantly more vulnerable to mobile radiations induced oxidative stress due to the intense consumption of oxygen, and high concentrations of polyunsaturated fatty acids in neuronal membranes. Cognitive and behavioral dysfunctions are being the most apparent CNS impairments reported in both humans (Babadi-Akashe et al., 2014; Soderqvist et al., 2015) and animals (Zhao et al., 2012) following long-term mobile radiations exposure. We analyzed the hippocampus tissue due to its role in several cognitive aspects linked to learning and memory functions (Bast, 2007). In the previous studies neurobehavioral impairments like modifications in neuronal electrophysiological activity (Roggeveen et al., 2015), increases in the

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permeability of the blood–brain barrier (BBB) (Sırav and Seyhan, 2016), disorders in neurotransmitter release (Noor et al., 2011;), alterations in brain structure (Saikhedkar et al., 2014) and cognitive deficits (Megha et al., 2012) have been observed following microwave radiations exposure.

In the present study, we have demonstrated and attempted to establish a correlation between the influences of oxidative stress associated redox glutathione metabolism, cell damage in the hippocampus and related altered working memory following mobile emitted EMR exposure.

2. Material and Methods

2.1. Animals

The study was conducted on male Wistar rats (weighing 120-150 g) obtained from departmental animal house facility. Animals were kept in polypropylene cages containing dust- free clean husk bedding. Continuous supply of food and water was ensured. Animal room was maintained in a controlled temperature (25 ± 2 °C) and humidity (50–65%) with a fixed 12:12 h light-dark cycle. All the experimental studies were carried out as per the guidelines of CPCSEA and the procedures were pre-approved by the Institutional Animal Ethics Committee, Jiwaji University, Gwalior, India (1854/GO/Re/S/16/CPCSEA/IAEC/JU/24).

2.2. Exposure system

The electromagnetic radiations were produced by a dipole antenna designed by the electronics department of Madhav Institute of technology and Science (MITS), Gwalior. Exposure system consists of two antennae (receiver antenna and transmitting antenna), signal generator, a spectrum analyzer, a direct current (DC) regulator (Fig. 1/A-B). During exposure, rats were restrained in a box (L-100 cmXB-70cmX H-40 cm) closed box with 1 cm diameter hole to facilitate breathing. Animal cage kept at a distance of 100 cm from source box can hold four rats and two such boxes can be placed within the box. An internal antenna connected to signal generator (Rohde and Schwarz GmbH and co. Germany) for transmitting signal at 900 frequencies at power (1 mW). These transmitted signals were received by an antenna which was further measured with spectrum analyzer (ROHDE & SCHWARZ FS315) to track the receiving signals. The average whole-body Specific absorption rate (SAR) was measured on the basis of electric field and the tissue density as mentioned in our previous study (Sharma et al., 2019). The average localized SAR for brain region is 0.231 W/kg at a maximum power density (7.737 μW/m2). The calculation formula used for measurement is-

$$SAR = \sigma E^2/2\rho \ (W/Kg)$$

Where,

- σ - electrical conductivity
- ρ - sample density (1000 kg m^3)
- E - root mean square of induced electric field strength (V/m) in tissue

2.3. Experimental Design

Animals were allocated into four groups: group I (n = 6), Sham exposed without near source of electromagnetic radiation but kept under same conditions as that of exposed group; and group II-IV (n = 6) were exposed to 900 MHz MW radiation at frequency 900 MHz, SAR 0.231 W/Kg for 1, 2 and 4 h/day respectively over a period of 90 days.

After the radiation exposure, spatial working memory assessment was done by using T-maze. On completion of the study duration, further animals were sacrificed and hippocampus was dissected out, rinsed in a cold 0.9% sodium chloride solution, placed in -80 °C for further assessments.

2.4. Spatial working memory test by T-Maze

Spatial working memory as observed by T-maze. In this study, animals were trained to find the baited arm (arm having eatable reward) based on their memory of previously visited arm (Nasuti et al., 2013). T maze is a metallic apparatus having 'T' shape i.e., one long start arm (60 × 10 x 10 cm) connected to two identical reward arms (45 × 10 x 10 cm). Preinstalled Any Maze software version 4.82 (Stoelting, USA) was

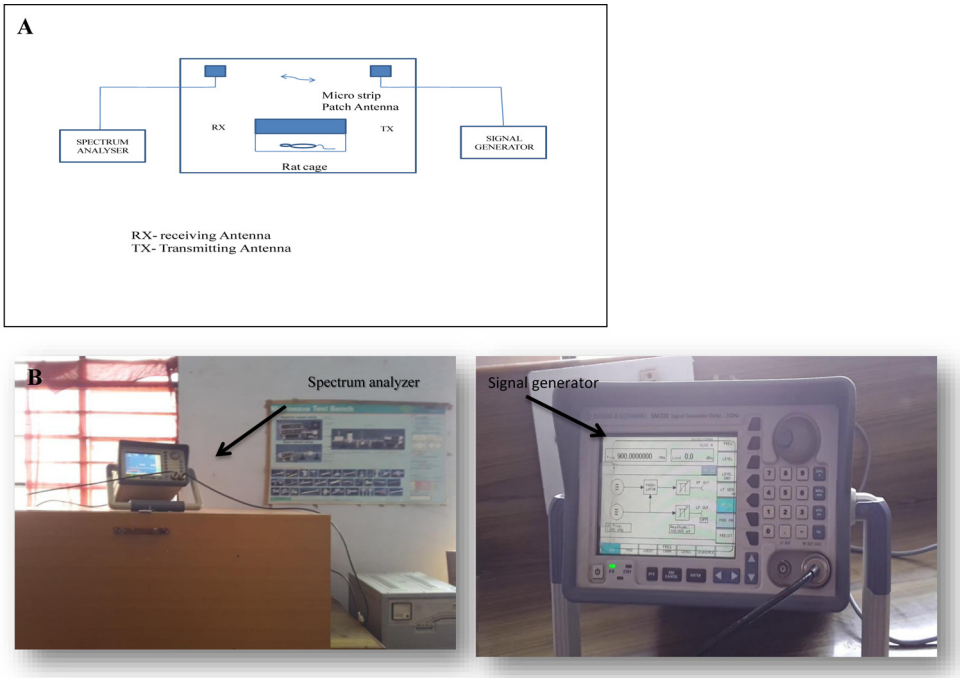


Fig. 1. A-B. Demonstrated the layout and schematic design of exposure setup.

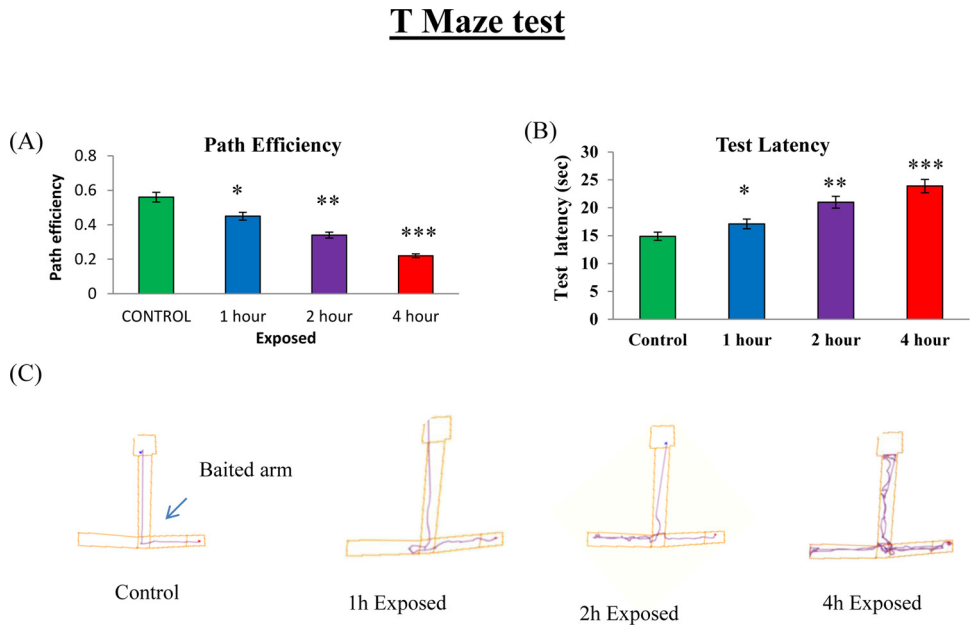


Fig. 2. Graphs and track report showing reduced spatial working memory of EMR exposed animals: Exposed animals were significantly taking more time (A) to reach the baited arm and presented poor path efficiency (Band C). Values are mean ± S.E; N = 6; *** P < 0.001, ** P < 0.01* P < 0.05 vs Control.

used to track the detail with the help of a camera. Animals those were not capable to find out the baitd arm in the provided time duration (5 min) were excluded from the study. Animals were acclimatized for 3 consecutive days 4 trials per day to learn the baited arm. The trial was accomplished when the animal reached the baited arm or after 5 min had elapsed. Animals were tested for 6 trials successively to assess the time duration animal acquired to choose the rewarded arm and path efficiency to reach this arm at the final test day.

2.5. Biochemical assessments

2.5.1. Oxidative stress Marker (LPO, SOD, CAT)

Thiobarbituric acid reactive substances (TBARS) were estimated for the determination of lipid Peroxidation in brain tissue as described by Sharma and Krishnamurthy (1968). The values of LPO in the brain were expressed as n mole of MDA/mg protein. Catalase (CAT) activity was estimated following Aebi (1984). Specific activity is expressed as l mole H_2O_2 decomposed min^{-1} mg^{-1} protein. Total superoxide dismutase (SOD) activity was estimated by measuring the inhibition of the auto-oxidation of epinephrine according to the method described by Misra and Fridovich (1972).

2.5.2. Redox Enzymes

Brain reduced glutathione was estimated using 5,5-dithiobis-2- nitrobenzoic acid (**Himedia-RM1677-5G**) (Brehe and Burch, 1986). The rate of conversion of NADPH to $NADP^+$ through the enzyme was measured for estimation of Glutathione reductase activity in brain tissue according to the method used by Tayarani et al. (1989) and absorbance change was recorded at 340 nm. Glucose-6-phosphate dehydrogenase (G-6-PDH) activity was estimated following the method described by Askar et al. (1996). Activity was expressed as µmol NADPH formed min^{-1} mg^{-1} protein.

2.6. Assessment of Cholinesterase activity

An activity of acetylcholinesterase (AChE) in the brain was carried out according to the method of Ellman et al. (1961) using acetylcholine iodide (**Himedia- RM770-1G**) as substrate and optical density was recorded immediately at ʎ 412 nm.

The protein content in the brain was measured by method Lowry et al. (1951) with bovine serum albumin (**Sigma A7906-10G**) as the standard.

2.7. Histopathological study

For histological study tissues were fixed in 10% formalin. After fixation, tissues were carefully washed with water and dehydrated with a graded series of ethyl alcohol. Then, the tissues were cleared in toluene and infiltrated in Paraplast (Sigma, m. p.56–58 °C) for proper impregnation of wax. Tissue blocks were made in paraffin and serial coronal sections of the hippocampus were cut at a thickness of 10 µm using microtome.

2.8. DNA damage study

DNA damage was evaluated by single cell gel electrophoreses (comet assay) using the method of Singh et al. (1998). The comet moment (diameter of the nucleus plus migrated DNA) was calculated from 50 counted cells and was expressed in micrometer.

2.9. Statistical analyses

Results were statistically analyzed with Sigma Stat software 3.5 versions and were reported as mean ± SEM. The significance of the differences between mean values were determined by one-way analysis of variance (ANOVA) followed by the Student's t-test according to the Snedecor and Cochran (1994). **Differences among groups were analyzed by one way analysis of variance (ANOVA). Student's t test was used for pair wise comparisons among groups.** The threshold for statistical significance was set at $p < 0.05$. In figures * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

3. Results

3.1. Effect of EMR on cognitive ability

On T-maze task mobile radiation exposed animals performed poorly while exploring the baited arm (Fig. 2). The time duration was significantly increased [F (3,23) = 33.612; P ≤ 0.001](Fig. 2/A) and path efficiency [F (3,23) = 48.916; P ≤ 0.001] (Fig. 2/B) was reduced in

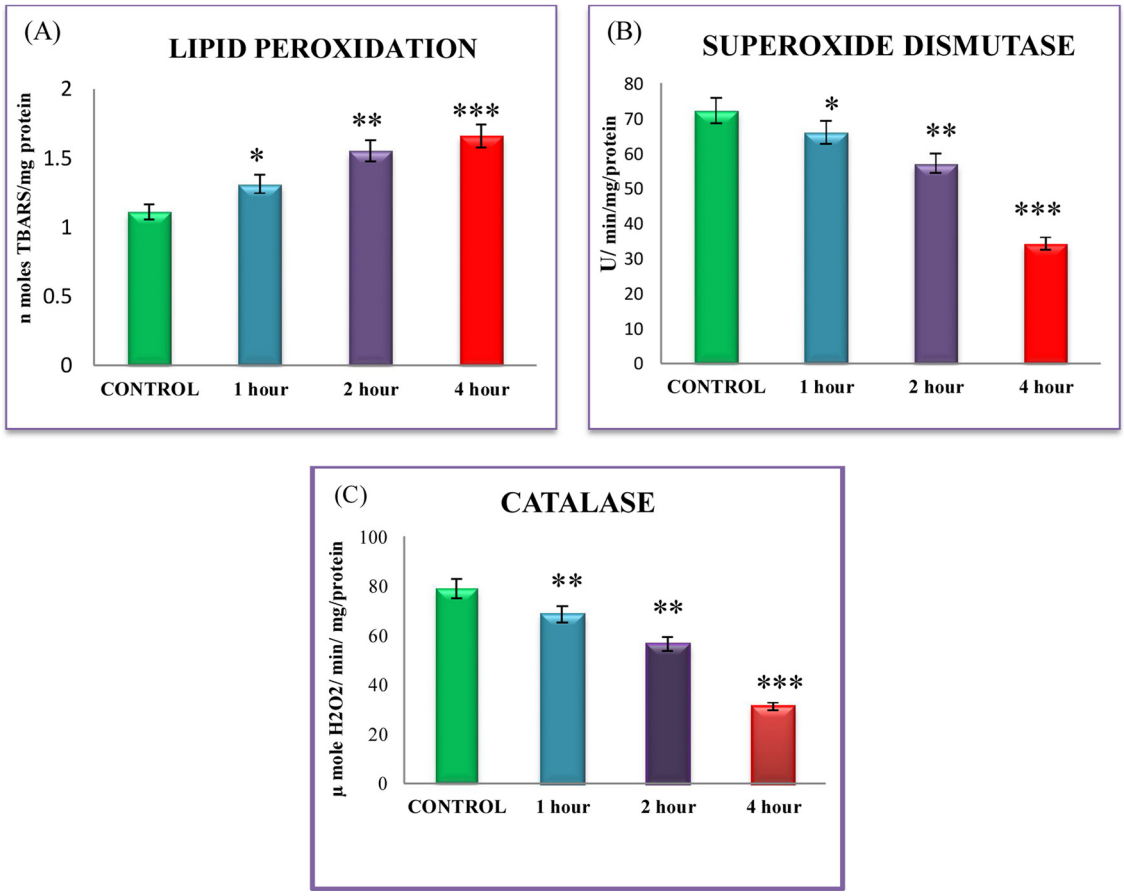


Fig. 3. Effects of EMR on oxidative stress markers in hippocampus. Values are mean ± S.E; N = 6, *** P < 0.001, ** P < 0.01* P < 0.05 vs Control.

mobile radiation exposed animals in comparison to the controls showing altered effect of mobile exposure on spatial working memory in time- dependent manner.

3.2. Effect of microwave exposure on oxidative stress Markers (LPO, SOD, and CAT)

Fig. 3/A indicated significantly elevated neuronal LPO level following microwave radiation exposure. Three-month exposure of MWR was able to induced lipid peroxidation in brain tissues significantly. The LPO induction was found to be time dependent. Elevation in the LPO level was lowest in the control and highest at 4 h exposed group. Exposure duration for 2 h and 4 h caused more peroxidative damage in the brain cells as compared to 1 h exposure duration for 90 days. Fig. 3/B and /C shown activity of SOD and catalase was found to be depleted in EMR exposed animals in comparison to the control for 1 h, 12 h and 4 h for 90 days exposure duration which indicates susceptibility of the brain against ROS produced by EMR. More prominent depletion was observed in 2 and 4 hours ($P \leq 0.001$) exposed animals as compared to 1 h ($P \leq 0.01$) exposed group Analysis of variance showed significant result at 1% level.

3.3. Effect of microwave exposure on redox status of brain tissue

To determine whether the increased susceptibility of LPO in EMR exposed rats was associated with the depletion of redox enzymes. The concentration of GSH, GR, GPx, G-6-PDH, and GST was also determined in the brain tissue of experimental rats (Fig. 4/B). Data depict a considerable slump in the reduced glutathione contents of the brain after EMR exposure. Maximum reduction was observed in 4 hour ($P \leq 0.001$) exposed group as compared to 2 ($P \leq 0.01$) and 1 ($P \leq 0.01$) hour exposed group respectively.

3.3.1. Glutathione Peroxidase

Electromagnetic radiation emitted from mobile phones inhibited the enzymatic activity of GPx in the brain of all exposure duration (Fig. 4/B). While maximum reduction was observed in 4 h duration ($P \leq 0.01$) compared to 2 h, and 1 h ($P \leq 0.05$) exposed groups.

3.3.2. Glutathione Reductase

Fig. 4/**C depicted effect of EMR on GR activity in rat's brain. The activities of GR decreased significantly at different duration i.e., 1 h, 2 h and 4 h exposed rats, compared with the control. At the highest duration, the maximum inhibition was recorded as compared to 1 h and 2 h duration.**

3.3.3. Glutathione-S-Transferase

Rat brain tissue exposed to EMR exposure had significantly depleted GST activity when compared to the control. The depletion of GST appeared to be duration dependent; the highest duration of mobile radiation exposure caused more depletion of GST in the brain cells, followed by 1 hour and 2- hour duration when to the control (Fig. 4/D).

3.3.4. Glucose-6-phosphate dehydrogenase

G-6-PDH activity was significantly suppressed in the brain of EMR exposed rats relative to the control. However, maximum reduction was observed in 4 hours treated group ($p > 0.001$) as compared to 1 hour and 2 -h duration. A reduction in G-6-PDH enzyme activity after EMR exposure may affect the rate of NADPH production and thus affects redox reactions in the cell (Fig. 4/E).

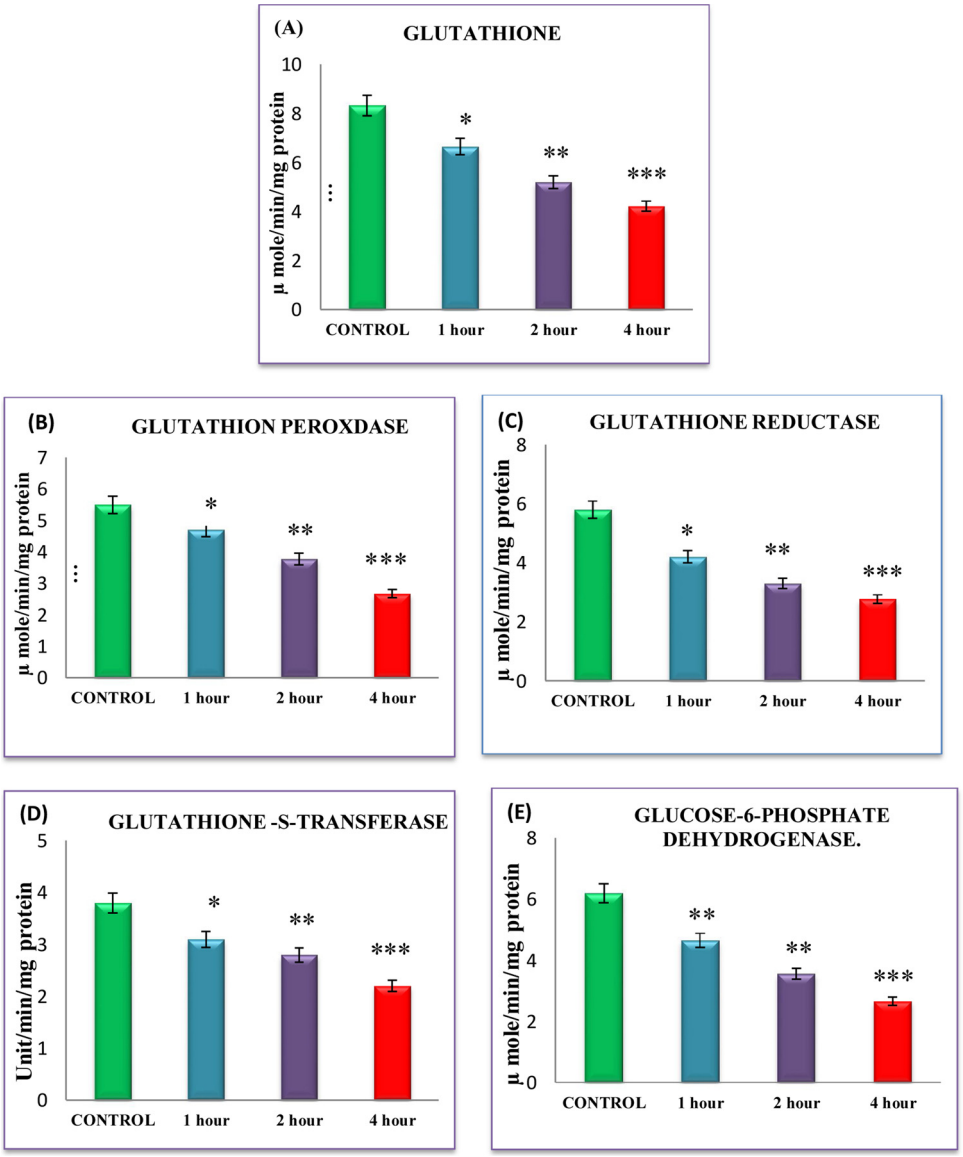


Fig. 4. Effects of EMR on antioxidant enzyme status in hippocampus. Values are mean ± S.E; N = 6, *** P < 0.001, ** P < 0.01* P < 0.05 vs Control.

3.4. Effect of microwave exposure on the cholinesterase activity

3.4.1. Acetyl Cholinesterase activity

We assessed the effect of exposure on neurotransmitter acetylcholinesterase in the brain. Fig. 5 reported AChE activity in the hippocampus of the exposed and control group. The toxic effect of radiation was clear as seen by significant inhibition of AChE activity in the hippocampus of the brain. The enzymatic activity decreases significantly in at gradual manner with time duration. Maximum depletion in activity observed at the higher duration of mobile radiation exposure. Depletion in cholinesterase activity during mobile exposure clearly indicates mobile radiation- induced neurotoxic effects.

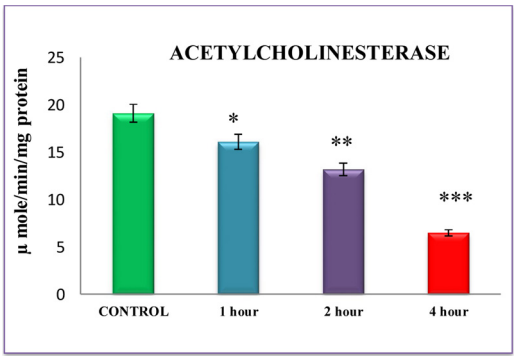


Fig. 5. Effect of EMR on cholinergic activity in brain hippocampus. mean ± S.E; N = 6, *** P < 0.001, ** P < 0.01* P < 0.05 vs Control.

3.5. Effect of microwave exposure on the DNA damage

We assessed the time course of microwave radiation genotoxicity in the brain of animals using the comet assay for the biomonitoring of DNA integrity. The alkaline comet assay (single-cell gel electrophoresis) is a sensitive method for measuring DNA strand breaks. As many cells are measured (e.g. 25-30 cells per object slide), the statistical distribution of the individual cell measurements represents the amount of DNA damage/alteration of the cell sample (Fig. 6). A statistically significant qualitative and quantitative increase in the oxidative DNA damage was observed in the brain of rats exposed to MWR as was evident by an increase in tail movement (Fig. 7/A), DNA damage (Fig. 7/B), and tail length (Fig. 7/C).

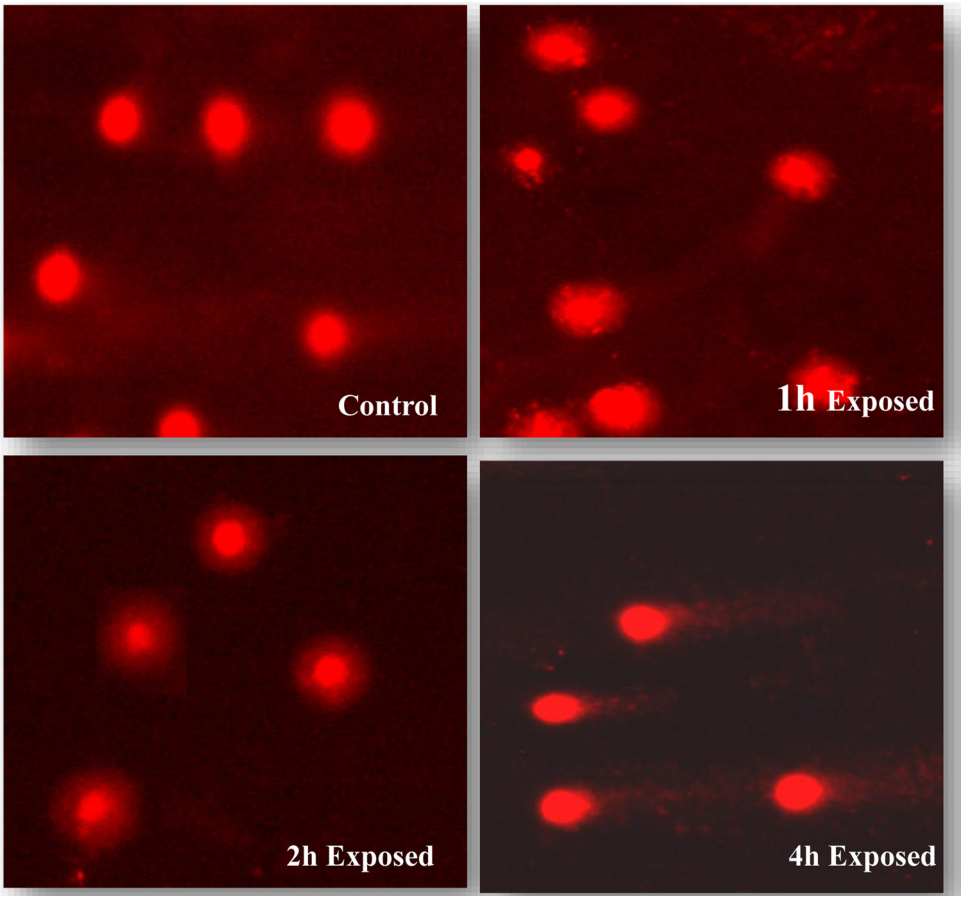


Fig. 6. Micrograph of control showing damage DNA in brain cells. Slide shows ethidium bromide stained nuclei after EMR and analyzed using florescence microscopy (Magnification 10 X) DNA migration was analyzed by image analysis.

3.6. Effect of microwave exposure on cellular damage

Fig. 8**(A-C)**: Brain of control rats showed normal neurons with a central nucleus and granular cytoplasm. Fig. 8**(D-L)** showed cerebral atrophy, vacuolization and degenerated pyramidal cells in the hippocampus of the brain of EMR exposed rats however these degenerations are more prominent in 2 and, 4 h exposed rat hippocampus with respect to 1 h EMR exposed rats brain hippocampus.

4. Discussion

The main purpose of this study is to find out a possible mechanism of cognitive alteration due to long term mobile use. Previously, several studies have reported the alterations in cognitive alteration especially for learning ability following long term electromagnetic exposure (Lu et al., 2012; Hao et al., 2013; Narayanan et al., 2015) while the possible mechanism behind these alterations still unclear, Therefore, we have investigated the effect of EMR in terms of antioxidant level, enzymological activity, biochemical parameters, genotoxic and histopathological observations in brain tissue in order to establish the possible systematic relation between these factors contributing in alter behavioral activity.

Considerably, oxidative damage is playing an important role in the etiology and progression of pathophysiology associated with EMR exposure in brain cells (Sahin et al., 2016; Guler et al., 2015; Çelik et al., 2016; Dasdag and Akdag, 2016). Similar manifestation of augmented free radicals in EMR exposed rats was measured in term of malondialdehyde level, an indicator of lipid peroxidation was reported along with compromise intracellular redox enzyme status in brain cells. Depletion of GSH and associated enzymes glutathione peroxidase, Glutathione reductase, glutathione-s-transferase and, G6PDH was observed following EMR exposure. GSH could directly diminish a number of reactive oxygen species (ROS) and is oxidized to GSSG in this process. A decrease in the levels of GSH in the brain of rats in response to mobile radiation exposure, observed in the present study, clearly shows the oxidizing conditions of the brain. The depletion of glutathione redox could be associated with increased ROS or decrease the availability of substrate (reduced glutathione). The redox boundaries limit each cellular stage perceptibly depend on cell type. Less “flexible” cells, like neurons cells, probably have a narrower redox range determining the steady-state condition. Hippocampus is one of the parts of the brain region that is exceptionally susceptible to neuronal loss during mild stress (Sharma et al., 2017). Therefore, redox imbalance plays a key role in the onset and progression of many neurological disorders (Kulak et al., 2012). **Similar to our study, researchers have reported increased lipid peroxidation and decreased antioxidant levels in different tissues due to mobile radiation exposure.** Ikinci et al. (2016) **documented that exposure to 900 MHz EMF has been affecting the spinal cord, leading to the pathological changes. An another study has also reported that mobile phone radiation exposure may induce oxidative damage mediated by increased lipid peroxidation and decreased antioxidant level during epileptic activity in mouse brain (**Esmekaya et al., 2016**).**

Manifestation of EMR induced oxidative stress has been believed to cause DNA damage and increased suceptibility for cancer in brain cells (Yakymenko et al. 2015; Akdag et al., 2016; Alkis et al., 2019**) as DNA damage was detected in various cancerous tissues (**Young, 2007**). The various reports on DNA damage following EMF exposure were not univocal, as some of them has showed no effect (**Verschaeve, et al., 2006**), while others presented the incidence of**

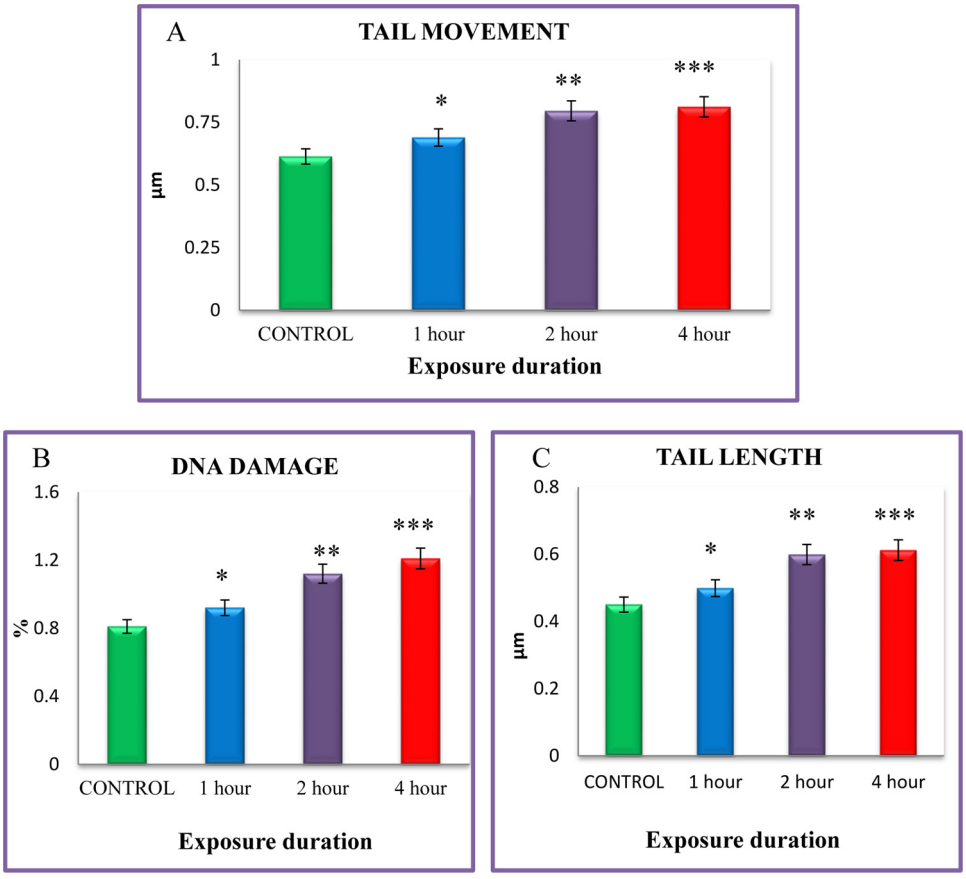


Fig. 7. Level of Tail length, Tail movement and DNA damage in brain of control and EMR exposed rats. Values are mean ± S.E; N = 6; *** P < 0.001, ** P < 0.01* P < 0.05 vs. Control.

DNA damge (Gandhi et al., 2015; Akdag, et al., 2018**).** Sahin et al. (2016) **reported the increased oxidative DNA damage to brain after 10 days of exposure with the decreased oxidative DNA damage following 40 days of EMR exposure.**

Redox changes could drastically alter Neurophysiologic alterations which were observed in the current study by examining histopathological changes. An increase in the number of distorted and darkly stained nuclei and decrease in the total number of neuron in the hippocampus region, as results of long term mobile phone exposure represents degenerative changes in hippocampal neurons. **Additionally, some pyramidal cells with deeply stained nuclei with intranuclear vacuolation and pyramidal cells with distorted irregular shape clearly depicted the degeneration during EMR exposure. The presence of pathological changes at light microscopic levels shows that long term EMR exposure also impairs the morphology of the hippocampus. The amplitude of tissue degeneration reported in this study are comparable with previous studies investigating the effects of 900 MHz EMF on various other tissues (including the spinal cord, cerebellum, testis, liver and heart) (**Hancı et al., 2013; Ikinci et al., 2016; Odaci et al., 2015; Topal et al., 2015; Turedi et al., 2015**).**

Furthermore, a significant alteration in cholinergic functions as observed in the present study could be a consequence of oxidative stress and morphological neurodegenerative alterations (Cardozo et al., 1994). Depleted AChE or cholinergic activity may indicate a brain at risk or in the preclinical stage of dementia (Shen, 1996). The reduction of cholinergic markers is a critical component of memory deficits (Terry and Buccafusco, 2003). These altered neurotransmitters also caused an impact on the functional status (neurobehavioral) in the study. Moreover, altered cholinergic activity in the hippocampus could be proposed as the neurological basis of behavioral alterations. In our, study we found that EMR-exposed animals were poorly performing on T maze task revealing a reduced working memory. These alterations in behavior may also be due to the direct inhibitory effect of EMR radiations on cholinesterase activity in the hippocampus. **As the hippocampus process storing and retaining of information in the learning route, therefore a negative impact of EMR exposure would possibly affecting the regulation and maintenance of the learning and memory (**Bas et al., 2009**). Recent study also demonstrated exposure of EMR induced several neurological disorders including depression (**Pall, 2016; Terzi et al., 2016**)**

The present collective evidence grounds the consideration that EMR induces (a) oxidative stress mediated by increased lipid peroxidation and depleted redox metabolism and (b) and depleted cholinesterase activity in the hippocampus (C) cellular degeneration in hippocampus. Later, the triangular associative interplay between these above mentioned three factors in the hippocampus results in memory deficits following mobile emitted radiations. More efforts are required to better elucidate the path physiological changes that underpin the progression and severity of brain disorders following mobile exposure.

Author statement

This manuscript has not been published and is not under consideration for publication elsewhere. All the authors have read the manuscript and have approved this submission.

Ethical statement

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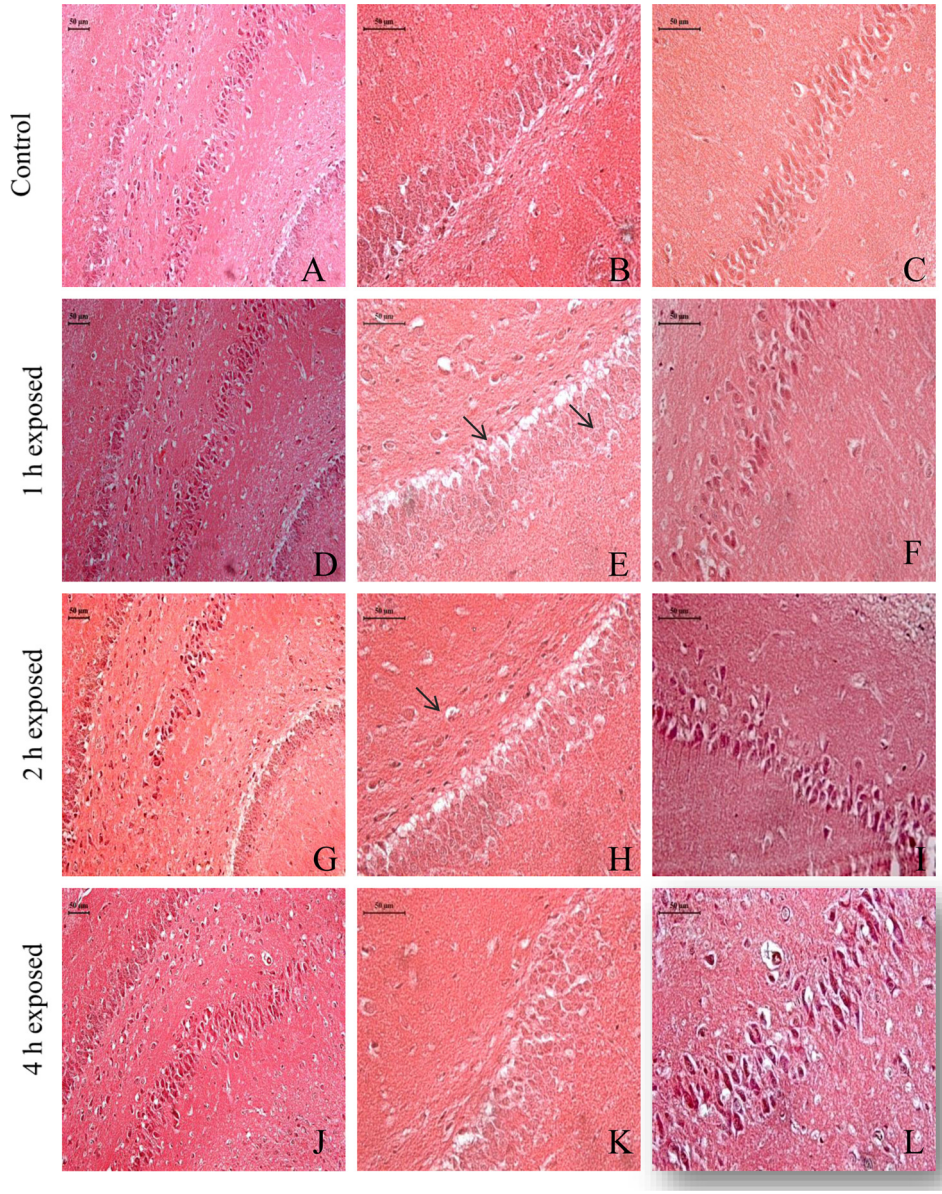


Fig. 8. Cellular degeneration hippocampus following EMR exposure. Histological H& E staining depicted cellular degeneration in Brain hippocampus, neurons are loosely arranged. There is also a significant shrinkage of neurons with evidence of karyopyknosis (D-L While in control photomicrograph of hippocampus, showing normal neurons (arrow) (A-c),) (X 200, 400). Scale bar = 50 μm.

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